Amendments to the Specification:

Please add the following new paragraphs prior to the "Background of the Invention" section of the application beginning on page 1:

Related Applications

The present application is a continuation-in-part of U.S. Application No. 09/652,345, filed August 31, 2000, which claims the benefit of U.S. Application No. 60/151,802, filed August 31, 1999.

This invention was made with Government Support under Contract Number MH-49469 awarded by the National Institutes of Health. The Government has certain rights in the invention.

Please insert the following paragraphs after the paragraph at page 3, lines 14-28.

In one aspect, the invention features a method for identifying a subunit specific modulator of the N-methyl-D-aspartate (NMDA) receptor. The method includes, for example: a) providing a plurality of NMDA receptors which differ in their subunit identity; b) contacting the NMDA receptors of step a) with a neurotransmitter recognition site ligand in the presence and absence of a candidate modulator; and c) assaying for receptor activity following step b), wherein an increase or decrease in activity in at least one, but not all members of the plurality of NMDA receptors, in the presence but not the absence of a candidate modulator, is an indication that the candidate modulator is a subunit specific modulator.

The method can further include comparing the subunit identity of the subset of the NMDA receptors to determine the subunit specificity of the candidate modulator.

In one embodiment, the plurality of NMDA receptors have identical NR2 subunits, and differ in their NR1 subunits. In one embodiment, the identical NR2 subunits are selected from the group consisting of NR2A, NR2B, NR2C, and NR2D. In one embodiment, at least one of the NR1 subunits is a natural isoform selected from the group consisting of $NR1_{000}$, $NR1_{001}$, $NR1_{010}$,

$NR1_{011}$, $NR1_{100}$, $NR1_{101}$, $NR1_{110}$, and $NR1_{111}$. In one embodiment, at least one of the NR1 subunits contain an α exon encoded protein domain.

In one embodiment, at least one of the NR1 subunits is a chimeric isoform. In one embodiment, at least one of the NR1 subunits is an isoform point mutant. In one embodiment, the point mutant contains at least one point mutation at a residue which corresponds to residue 182, 193, 202, 233, or 252 of $NR1_{011}$.

In one embodiment, the isoform point mutant is a penta-mutant with the amino acid substitution mutations which correspond to mutations R182A, K193A, K202A, R233A, and R252A of $NR1_{011}$. In one embodiment, the isoform point mutant contains an α exon encoded protein domain and has point mutations within that domain.

In one embodiment, the plurality of NMDA receptors have identical NR1 subunits, and differ in their NR2 subunits. In one embodiment, the identical NR1 subunits are an isoform selected from the group consisting of $NR1_{000}$, $NR1_{001}$, $NR1_{010}$, $NR1_{011}$, $NR1_{100}$, $NR1_{101}$, $NR1_{110}$, and $NR1_{111}$. In one embodiment, the identical NR1 subunits contain an α-exon encoded protein domain.

In one embodiment, the identical NR1 subunits are a chimeric isoform. In one embodiment, the identical NR1 subunits are an isoform point mutant which contains an α exon encoded protein domain and has point mutations within that domain. In one embodiment, the identical NR1 subunits contain an α exon encoded protein domain.

In one embodiment, the identical NR1 subunits are point mutants which contain at least one point mutation at a residue which corresponds to residue 182, 193, 202, 233, or 252 of $NR1_{011}$. In one embodiment, the identical NR1 subunits are a penta-mutant with the amino acid substitution mutations which correspond to mutations R182A, K193A, K202A, R233A, and R252A of $NR1_{011}$.

In one embodiment, at least one of the the NR2 subunits is an isoform selected from the group consisting of NR2A, NR2B, NR2C, and NR2D.

In one embodiment, at least one of the NR2 subunits is a chimeric isoform.

In one embodiment, the chimeric isoform contains a.a. 534-870 of NR2B. In one embodiment, the chimeric isoform contains amino acid 548-892 of NR2D. In one embodiment, the chimeric isoform contains amino acid 703-870 of NR2B.

In one embodiment, at least one of the NR2 subunits is an isoform point mutant.

In one embodiment, the assaying step is with an oocyte expression system.

In one embodiment, the neurotrasmitter recongition site ligand is an agonist. In one embodiment, the agonist is selected from the group consisting of NMDA, glutamate, and glycine.

In one embodiment, the neurotrasmitter recongition site ligand is an antagonist.

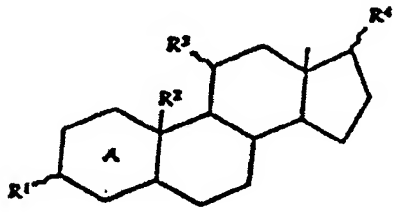
In one embodiment, the candidate modulator is a steroid based molecule.

In one embodiment, the candidate modulator is a non-steroid based molecule.

In one embodiment, the candidate modulator is obtained from a library of small molecules.

In one embodiment, the candidate modulator is a known neuromodulator.

In another aspect, the invention features a method for inhibiting N-methyl-D-aspartate glutamate receptor mediated ion-channel activity in an individual in need thereof. The method includes, for example administering an effective amount of a compound represented by the following structural formula:

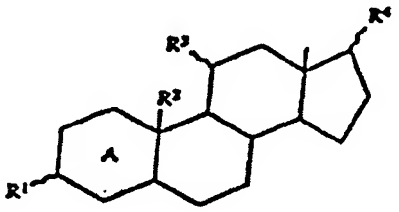


wherein: ring A has 0-3 double bonds; R^1 is -OH, =O, or a negatively charged group; R^2 is -H, -CH_3, or is absent when ring A has three double bonds; R^3 is -H, OH, =O, or -OR'; R^1 is an aliphatic or aromatic group; and R^4 is -OH, =O or -$COCH_3$.

In one embodiment, R^1 is either hemioxylate, hemisuccinate, or hemiglutarate.

In one embodiment, the compound is selected from the group consisting of pregnanolone hemioxylate (3α5βHO), pregnanolone hemisuccinate (3α5βHS), and pregnanolone hemiglutarate (3α5βHG). In one embodiment, the effective amount is a concentration of from about 1 to about 500 μM. In one embodiment, the effective amount is from about 50 to about 250 μM.

In another aspect, the invention features a method for inhibiting the toxic effects associated with activation of the N-methyl-D-aspartate receptor in neurons in an individual in need thereof, comprising administering an effective amount of a compound represented by the following structural formula:



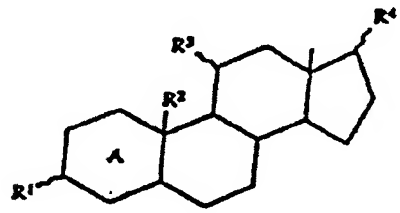
wherein: ring A has 0-3 double bonds;R^1 is -OH, =O, or a negatively charged group; R^2 is -H, -CH_3, or is absent when ring A has three double bonds; R^3 is -H, OH, =O, or -OR'; R^1 is an aliphatic or aromatic group; and R^4 is -OH, =O or -$COCH_3$.

In one embodiment, R^1 is either hemioxylate, hemisuccinate, or hemiglutarate.

In one embodiment, the compound is selected from the group consisting of pregnanolone hemioxylate (3α5βHO), pregnanolone hemisuccinate (3α5βHS), and pregnanolone hemiglutarate (3α5βHG). In one embodiment, the effective amount is a concentration of from about 1 to about 500 μM. In one embodiment, the effective amount is from about 50 to about 250 μM.

In one embodiment, the neurons are selected from the group consisting of hippocampal cells and spinal cord cells.

In another aspect, the invention features a method for reducing neuronal cell death resulting from L-glutamate activation of the N-methyl-D-aspartate receptor in an individual in need thereof, comprising administering an effective amount of a compound represented by the following structural formula:

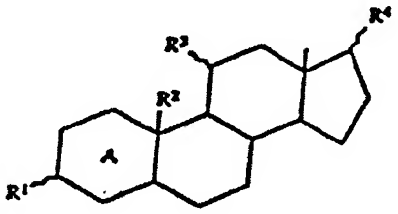


wherein: ring A has 0-3 double bonds; R^1 is -OH, =O, or a negatively charged group; R^2 is -H, -CH_3, or is absent when ring A has three double bonds; R^3 is -H, OH, =O, or -OR'; R^1 is an aliphatic or aromatic group; and R^4 is -OH, =O or -$COCH_3$.

In one embodiment, R^1 is either hemioxylate, hemisuccinate, or hemiglutarate. In one embodiment, the compound is selected from the group consisting of pregnanolone hemioxylate (3α5βHO), pregnanolone hemisuccinate (3α5βHS), and pregnanolone hemiglutarate (3α5βHG).

In one embodiment, the effective amount is a concentration of from about 1 to about 500 μM. In one embodiment, the effective amount is from about 50 to about 250 μM.

In another aspect, the invention features a method for treating a disease selected from the group consisting of neuropathic pain, drug withdrawal/dependency, epilepsy, glaucoma, chronic neurodegenerative diseases, amyotrophic lateral sclerosis, anxiety disorders, brain cell death, ischaemia, stroke, and trauma in an individual when said disease results from agonist induced NMDA receptor activation comprising administering to the individual an effective amount of a compound represented by the following structural formula:



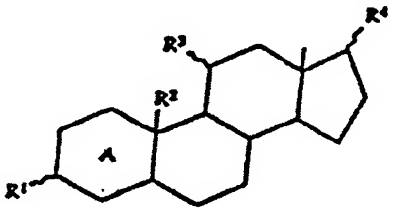
wherein: ring A has 0-3 double bonds; R^1 is -OH, =O, or a negatively charged group; R^2 is -H, -CH_3, or is absent when ring A has three double bonds; R^3 is -H, OH, =O, or -OR'; R^1 is an aliphatic or aromatic group; and R^4 is -OH, =O or -$COCH_3$.

In one embodiment, R^1 is either hemioxylate, hemisuccinate, or hemiglutarate.

In one embodiment, the compound is selected from the group consisting of pregnanolone hemioxylate (3α5βHO), pregnanolone hemisuccinate (3α5βHS), and pregnanolone hemiglutarate (3α5βHG).

In one embodiment, the effective amount is a concentration of from about 1 to about 500 μM. In one embodiment, the effective amount is from about 50 to about 250 μM.

In another aspect, the invention features a method for inhibiting the excitatory L-glutamate-mediated synaptic activity in an individual in need thereof, comprising administering to the individual a compound represented by the following structural formula:



wherein: ring A has 0-3 double bonds; R^1 is -OH, =O, or a negatively charged group; R^2 is -H, -CH_3, or is absent when ring A has three double bonds; R^3 is -H, OH, =O, or -OR'; R^1 is an aliphatic or aromatic group; and R^4 is -OH, =O or -$COCH_3$.

In one embodiment, R^1 is either hemioxylate, hemisuccinate, or hemiglutarate.
In one embodiment, the compound is selected from the group consisting of pregnanolone hemioxylate (3α5βHO), pregnanolone hemisuccinate (3α5βHS), and pregnanolone hemiglutarate (3α5βHG).

In another aspect, the invention features a N-methyl-D-aspartate (NMDA) receptor NR2 subunit polypeptide comprising a mutation within the region consisting of the fourth transmembrane domain and about 68 amino acids preceding the fourth transmembrane domain.

In one embodiment, the mutation is a substitution of one or more amino acids.

In one embodiment, the mutation is a deletion of one or more amino acids.

In one embodiment, the NR2 subunit is an NR2B subunit.

In one embodiment, an amino acid corresponding to Q812 of human NR2B is mutated.
In one embodiment, an amino acid corresponding to Q812 of human NR2B is mutated to K.

In one embodiment, the NR2 subunit is an NR2D subunit.

In one embodiment, the polypeptide does not contain mutations outside the region consisting of the fourth transmembrane domain and 68 amino acids preceding the fourth transmembrane domain.

In one embodiment, the NR2 subunit is a chimeric NR2B/NR2D subunit polypeptide comprising the fourth transmembrane domain and 68 amino acids of the extracellular domain preceding the fourth transmembrane domain of NR2B.

In one embodiment, the polypeptide comprises amino acids 750-839 of NR2B.

In one embodiment, the polypeptide comprises amino acids 750-870 of NR2B.

In one embodiment, the polypeptide comprises acids 818-870 of NR2B.

In one embodiment, the polypeptide comprises amino acids 1-774 and 892-1323 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 750-817 of NR2B.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 774-1323 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 774-892 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 774-863 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 863-892 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 842-892 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 774-842 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 548-1323 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 548-892 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 548-774 of NR2D.

In another aspect, the invention features an NMDA receptor NR2B/NR2D chimeric subunit polypeptide comprising amino acids 774-892 of NR2D.

In another aspect, the invention features an receptor NR2B subunit polypeptide comprising a deletion of amino acids 844-1482 (e.g., contains amino acids 1-843 of NR2B but with the signal sequence cleaved).

In one embodiment, there are no other mutations outside the region consisting of the fourth transmembrane domain and about 68 amino acids preceding the fourth transmembrane domain.

In another aspect, the invention features an isolated nucleic acid molecule encoding a polypeptide described herein.

In another aspect, the invention features a host cell comprising a polypeptide described herein. In one embodiment, the host cell is a *Xenopus* oocyte.

In another aspect, the invention features an antibody that binds to an NR2 subunit polypeptide described herein. In one embodiment, the antibody does not bind a non-mutated NR2 subunit polypeptide (e.g., a wild type NR2 subunit polypeptide).

In another aspect, the invention features a method for identifying a subunit specific modulator of the NMDA receptor. The method includes, for example:

(a) providing a plurality of NMDA receptors which differ in the identity of NR2 subunits;

(b) contacting the NMDA receptors of step (a) with a neurotransmitter recognition site ligand in the presence and absence of a candidate modulator; and (c) assaying for receptor activity following step (b), wherein an increase or decrease in activity in at least one, but not all members of the plurality of NMDA receptors, in the presence but not the absence of a candidate modulator, is an indication that the candidate modulator is a subunit specific modulator.

In one embodiment, each receptor of the plurality has an identical NR1 subunit.

In one embodiment, at least one of the NR2 subunits is an NR2 subunit described herein.

In one embodiment, the plurality comprises an NMDA receptor NR2B/NR2D chimeric isoform subunit polypeptide comprising the fourth transmembrane domain of NR2B and 68 amino acids of the extracellular domain preceding the transmembrane domain.

In one embodiment, the plurality comprises an NMDA receptor comprising an NR2C or NR2D isoform.

In one embodiment, the method further includes comparing the subunit identity of at least one NMDA receptor whose activity is increased or decreased to the members of the plurality of NMDA receptors whose activity is not increased or decreased to determine the subunit specificity of the candidate modulator.

In one embodiment, the assaying step (c) is with an oocyte expression system. In one embodiment, the neurotransmitter recognition site ligand is an agonist of an NMDA receptor. In one embodiment, the agonist is NMDA, glutamate, or glycine. In one embodiment, the neurotransmitter recognition site ligand is an antagonist of an NMDA receptor. In one embodiment, the candidate modulator is obtained from a library of small molecules.

In one embodiment, the candidate modulator is a known neuromodulator.

In another aspect, the invention features a method for making an NMDA receptor comprising: a) providing a nucleic acid encoding an NR1 subunit polypeptide; b) providing a nucleic acid encoding an NR2B/NR2D chimeric subunit polypeptide described herein. The method can further include the step of: c) expressing the nucleic acids in a host cell.

In one embodiment, the host cell is a *Xenopus* oocyte.

In one embodiment, the method further includes the step of: d) assaying activity of the NMDA receptor.

In another aspect, the invention features a method for increasing sensitivity of an NMDA receptor assay, the method comprising: a) providing an first NMDA receptor comprising a chimeric NR2B/NR2D subunit polypeptide described herein; b) assaying activity of the receptor. In one embodiment, sensitivity to PS modulation is increased. In one embodiment, sensitivity to spermine modulation is increased.

In another aspect, the invention features a method for making an NMDA receptor with increased sensitivity, the method comprising: a) providing a nucleic acid encoding an NR1 subunit polypeptide; b) providing a nucleic acid encoding an NR2B/NR2D chimeric subunit polypeptide, e.g., an NR2B/NR2D chimeric subunit polypeptide described herein.

In one embodiment, the method further includes expressing the nucleic acids in a host cell, thereby providing a first NMDA receptor.

In one embodiment, sensitivity to PS modulation is increased. In one embodiment, sensitivity to spermine modulation is increased.

In one embodiment, the method further includes the steps of: b) contacting the NMDA receptor of step a) with a neurotransmitter recognition site ligand in the presence and absence of a candidate modulator; and c) assaying for receptor activity following step b).

In one embodiment, the method further includes the steps of: d) contacting a second NMDA receptor with a neurotransmitter recognition site ligand in the presence and absence of a candidate modulator; e) assaying receptor activity following step d); and f) comparing receptor activity of the first NMDA receptor in the presence of the test compound to activity of the second NMDA receptor in the presence of the test compound.

In one embodiment, a difference in activity of the first NMDA receptor and the second NMDA receptor in the presence of the candidate modulator is an indication that the candidate modulator is a subunit-specific modulator.

In another aspect, the invention features a method for identifying a subunit specific modulator of the NMDA receptor comprising: a) selecting a chimeric NR2B/NR2D subunit polypeptide that has increased sensitivity to a neuromodulator (e.g., PS or spermine); b)

providing a first NMDA receptor comprising the NR2 subunit of a); c) contacting a NMDA receptor with a neurotransmitter recognition site ligand in the presence and absence of a candidate modulator; d) assaying for receptor activity following step c); e) comparing the activity of the NMDA receptor to the activity of a second NMDA receptor in the presence of the candidate modulator, wherein the second NMDA receptor does not have increased sensitivity to the neuromodulator, wherein a difference in activity of the first NMDA receptor and the second NMDA receptor is an indication that the candidate modulator is a subunit-specific modulator.

In one embodiment, the chimeric subunit polypeptide has increased sensitivity to PS. In one embodiment, the chimeric subunit polypeptide has increased sensitivity to spermine.

In another aspect, the invention features a method for increasing sensitivity of an NMDA NR2C or NR2D receptor, the method comprising: mutating residues within the region consisting of the fourth transmembrane domain and about 68 amino acids preceding the fourth transmembrane domain.

In one embodiment, the mutating comprises substituting amino acids. In one embodiment, amino acids from a corresponding region of NR2B are substituted for amino acids of the NR2C or NR2D subunit.

Please replace the paragraph beginning at page 3, line 32, as with the following amended paragraph:

Figure 2 is a compilation of graphical representations of data which indicate that [PS] pregnenolone sulfate (PS) inhibits [AMPA] α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptor function. Figures [1(A)]2(A) through [1(D)]2D are representative traces showing the inhibitory effect of 100 μM PS on kainate-induced currents of oocytes injected with (A) rat brain poly(A)$^+$ RNA, (B) GluR1 cRNA, (C) GluR3 cRNA, (D) GluR6 cRNA. The kainate concentration used in (A)-(C) was 100 μM, and in (D) was 10 μM. The *solid bar* represents the period of kainate (KA) application; the *open bar* indicates the period of PS exposure. Figure [1(E)]2E is a graph of relative current for the indicated Kainate concentration. The administration of PS (open symbols) is seen to decreases maximum kainate responses of GluR1 (, ○), GluR3 (■, □), and GluR6 (▲, Δ) receptors. Each *data point*

represents the mean of three experiments. *Error bars* represent standard error. *Smooth curve* was determined by nonlinear regression using the logistic equation applied to pooled data. Fitted parameters are (GluR1) I_{max} =1.0, EC_{50}=27 μM, n_H=1.54; (GluR1 + PS) I_{max}=0.17, EC_{50}=23 μM, n_H=0.9; (GluR3) I_{max} =1.15, EC_{50}=27 μM, n_H=1.44; (GluR3 + PS) I_{max} =0.33, EC_{50}=32 μM, n_H=1.93; (GluR6) I_{max} =1.0, EC_{50}=550 nM, n_H=1.1; (GluR6 + PS) I_{max} =0.69, EC_{50}=570 nM, n_H=1.2. Figure 2F is a graph of data showing the concentration dependence of PS inhibition of recombinant GluR1 (○), GluR3 (□), and GluR6 (▲) receptors. Results are expressed as percentage change in the peak 100 μM (GluR1 and GluR3) or 10 μM (GluR6) kainate-induced current in the presence of PS. Each *data point* is the mean of three experiments; *error bars* indicate S.E.M. For GluR1 and GluR3, *smooth curves* are derived from fits to the Michaelis-Menten equation, as fits to the logistic equation yielded Hill coefficients close to 1, with no significant improvement in sum of squares (F-test, $P > 0.05$). Fitted parameters are (GluR1) EC_{50}=43 μM, E_{max} = -99%; (GluR3) EC_{50}=12 μM, E_{max} = -90%. For GluR6, the smooth curve is derived from a fit to the logistic equation, as Michaelis-Menten fits were significantly poorer (F-test, $P < 0.05$). Maximum inhibition was constrained to 100%, as an unconstrained fit yielded an extrapolated maximum inhibition >100%. Fitted parameters are EC_{50}=80 μM, n_H=0.29.

Please replace the paragraph beginning at page 5, line 4, as with the following amended paragraph:

Figure 3 is a compilation of graphical representations of data which indicate that neuroactive steroids modulate NMDA responses of oocytes injected with specific NMDA receptor subunits. Figure [2(A)]3(A) indicates the potentiation of the 100 μM NMDA response by PS in oocytes injected with $NR1_{100}$ + NR2A cRNA. The *solid bar* indicates the period of NMDA exposure; the *open bar* indicates the period of PS exposure. Figure [2(B)]3(B) indicates inhibition of the 100 μM NMDA response by 3α5βS in oocytes injected with $NR1_{100}$ + NR2A cRNA. The s*olid bar* indicates the period of NMDA exposure; the *shaded bar* indicates the period of 3α5βS exposure. Figure [2(C)]3(C) indicates modulation of agonist efficacy by PS and 3α5βS in oocytes injected with $NR1_{100}$ + NR2A cRNA. PS (100 μM) increases the NMDA I_{max} but does not affect the EC_{50}. 3α5βS (100 μM) markedly reduces the NMDA I_{max} with little effect on EC_{50}. Peak NMDA responses are normalized to the peak 100 μM NMDA response.

Each *data point* represents the mean of three experiments. *Error bars* represent standard error. *Smooth curves* are derived from fits to the logistic equation. Fitted parameters are (control) EC_{50}=29 μM, E_{max}=1.14, n_H=1.43; (+PS) EC_{50}=30 μM, E_{max}=3.21, n_H=1.54; (+3α5βS) EC_{50}=15 μM, E_{max}=0.35, n_H=1.66. Figure [2(D)]3(D) is a graph indicating the concentration dependence of steroid modulation of the NMDA response of oocytes injected with $NR1_{100}$ + NR2A cRNA. NMDA (100 μM) and the indicated concentration of PS (●), 3β5βS (Δ), or 3α5βS (□) were applied simultaneously for 10 s. The peak NMDA-induced current is expressed relative to the average of control NMDA responses determined before application of steroid and after steroid washout. *Points* indicate mean of 6 (PS and 3α5βS), and 4 (3β5βS), experiments. *Error bars* indicate S.E.M. Smooth curves are derived from fits to the Michaelis-Menten equation, as fits to the logistic equation yielded Hill coefficients close to 1, with no significant improvement in sum of squares (*F*-test, $P > 0.05$). Fitted parameters are (for PS) EC_{50}=32 μM, E_{max}=4.43 (for 3α5βS) EC_{50}=41 μM, E_{max}=0.1; (for 3β5βS) EC_{50}=79 μM, E_{max}=0.26. (E) Concentration dependence for PS enhancement (●) and 3α5βS (Δ) and 3β5βS (□) inhibition of the NMDA response of oocytes injected with $NR1_{100}$ cRNA. NMDA (300 μM) and the indicated concentration of steroid were applied simultaneously. The peak NMDA-induced current is expressed relative to the average of control NMDA responses determined before application of steroid and after steroid washout. *Points* indicate mean of 6 (PS), 3 (3β5βS), and 3 (3α5βS) experiments. *Error bars* indicate S.E.M. Smooth curves are derived from fits to the Michaelis-Menten equation, as fits to the logistic equation yielded Hill coefficients close to 1, with no significant improvement in sum of squares (*F*-test, $P > 0.05$). Fitted parameters are (for PS) EC_{50}=26 μM, E_{max}=2.14; (for 3α5βS) EC_{50}=57 μM, E_{max}=0.02; (for 3β5βS) EC_{50}=144 μM, E_{max}=0.17.

Please replace the paragraph beginning at page 11, line 3, with the following amended paragraph.

Figure 13 contains graphical representations of data which compares 3α5βS and PS dose-responses for NR1/NR2A receptors. Presented are normalized current responses obtained from oocytes injected with ~~$NR1_{xxx}$/NR2A mRNAs.~~ (A): $NR1_{000}$/NR2A, $NR1_{100}$/NR2A; (B): $NR1_{001}$/NR2A, $NR1_{101}$/NR2A; (C): $NR1_{011}$/NR2A, $NR1_{111}$/NR2A mRNAs. The current was induced by coapplication of 10 mM glycine and 50 mM NMDA (for N-terminal insert lacking

NR1 isoforms, open symbols) or 80 mM (in B; 100 mM in A) NMDA (for N-terminal insert containing NR1 isoforms, closed symbols) and different concentration of 3a5bS (A) or PS (B). Error bars are S.E.M. Solid lines are drawn using equation $1+E\ max/(1+(EC_{50}/c)_n$ with parameters from Table 2 and 3.

Please replace the paragraph beginning at page 17, line 15, with the following amended paragraph.

Figure 29 contains graphical representations of data indicating the choice of NR2 subunit influences 3α5βS inhibition of the NMDA response. *A -D* are examples of traces obtained from oocytes previously injected with NR1/NR2A, NR1/NR2B, NR1/NR2C, or NR1/NR2D mRNAs, respectively. The *bar* indicates the period of drug application. Interval between consecutive current traces was 45 s. The receptors were activated by co-application of 10 μM glycine plus 80 μM NMDA (NR1/NR2A, A), 25 μM NMDA (NR1/NR2B, B and NR1/NR2C, C), or 10 μM NMDA (NR1/NR2D, D). *E* is concentration-response curves for 3α5βS effect on NR1/NR2 receptors. Data points are averaged values of normalized steady-state current responses from oocytes injected with NR1/NR2A (l, n=4), NR1/NR2B (□, n=3), NR1/NR2C (□, n=6) or NR1/NR2D (■, n=4) RNAs. Current responses are expressed relative to the current response in the absence of PS. Error bars represent SEM. *F* is a graph of data indicating dependence of 3α5βS effect on membrane potential. Points are averaged relative current obtained in the presence of 100 μM 3α5βS (l, NR1/NR2A, n=5; ○, NR1/NR2B, n=10) or 10 μM 3α5βS (■, NR1/NR2C, n=4; □, NR1/NR2D, n=10). G, I, K, and M [[is a]] are concentration response curves showing the effect of 3α5βS on glutamate. Data points are averaged normalized peak current responses to glutamate from oocytes injected with NR1/NR2A subunits obtained in the presence of 10 μM glycine and in the absence (l, n = 4) or presence (○, n = 3) of 100 μM 3α5βS. The data for each oocyte were normalized to standard current responses induced by co-application of 200 μM NMDA and 10 μM glycine. Concentration response data for glutamate alone is the same as in Fig. 27, and is repeated for comparison. H, J, L, and N indicate[[s]] the effect of 3α5βS on glycine concentration-response curve. Data points are averaged normalized peak current responses to glycine from oocytes injected with NR1/NR2A subunits obtained in

the presence of 10 μM glutamate and in the absence (s) or presence ($\triangle$) of 100 μM 3α5βS. The data for each oocyte were normalized to standard current responses induced by co-application of 200 μM NMDA and 10 μM glycine. Concentration response data for glycine alone is the same as in Fig. 28, and is repeated for comparison.

Please add the following Descriptions at page 19, line 18 of the specification, after the previous Descriptions of the Drawings:

Figure 33. Schematic diagram of chimeric and mutant NR2D/NR2B subunits. Portions of each molecule derived from NR2B have filled-in transmembrane domains and bold lines representing the loops between the transmembrane domains and N- and C-termini. Portions of each molecule derived from NR2D have open transmembrane domains and thin lines representing the loops between the transmembrane domains and N- and C-termini.

Chimera χ1 was constructed by replacing 96% of the cytoplasmic tail of NR2B with NR2D (amino acid residues 870 to 1482 of NR2B were replaced with amino acid residues 892 to 1323 of NR2D).

Chimera χ2 was constructed by replacing the region from amino acid residues 750 to 1482 of the NR2B subunit with the equivalent region of the NR2D subunit (774 to 1323).

Chimera χ3 was constructed by replacing amino acid residues 750 to 870 of the NR2B with the equivalent region of NR2D.

Chimera χ4 was constructed by replacing the region between 750 and 839 of NR2B with the corresponding region of NR2D. Chimera χ5 was constructed by replacing the region between 840 and 870 of NR2B with the corresponding region of NR2D.

Chimera χ6 was constructed by replacing the region from residues 750 to 818 of χ3 with the corresponding region of NR2B.

Chimera χ7 was constructed by replacing the region from residues 750 to 818 of NR2B with the corresponding region of the NR2D.

NR2BΔ was made by introducing a stop codon at amino acid residue 850 in the intracellular domain of NR2B. The only potential phosphorylation site left in the C-terminal domain after the truncation (Y843) was then substituted with alanine to construct NR2BΔ-Y843A.

Chimera χ8 was constructed by replacing the region from amino acid residues 774 to 892 of NR2D with the corresponding region of NR2B (residues 750 to 870).

Chimera χ9 was constructed by replacing residues 842 to 892 of NR2D with the corresponding region of NR2B (818 to 870). χ10 was constructed by substituting residues 774 to 841 of NR2D with residues 750 to 817 of NR2B.

The NR2D subunit was used as the backbone to construct two NR2D-NR2B chimeras: χ11 and χ12. First, the N-terminal region of the NR2D subunit (amino acid residues 1 to 548) was replaced with the corresponding region on the NR2B subunit (amino acid residues 1 to 524) to construct χ11. Next, the C-terminal region of χ11 (amino acid residues 892 to 1323) was replaced with the corresponding region on the NR2B subunit (amino acid residues 870 to 1482) to construct χ12.

Chimera χ13 was constructed by replacing residues 524 to 750 of NR2B with the corresponding region of NR2D.

Chimera χ14 was constructed by substituting residues 750 to 870 of NR2B with residues 774 to 898 of NR2D.

Chimera χ15 resembles χ14, except that the entire loop between the third and fourth transmembrane domains is derived from NR2D.

Figure 34. Residues in the region between amino acids 750 and 839 of the NR2B subunit are necessary for the potentiating effect of PS. A schematic representation of the wild-type NR2B, NR2D and the chimeras is on the left. The vertical bars correspond to the four hydrophobic domains. The contribution of the NR2B and the NR2D subunits to the chimeras is depicted in black and white respectively. The scales at the top indicate the amino acid residue numbers in the wild-type subunits. The percent increases in the NMDA/glycine response in the presence of 100 μM PS are indicated on the right. Holding potential: -70mV. Error bars indicate the standard error of the mean (SEM). Numbers adjacent to the error bars indicate the number of oocytes tested.

Figure 35. Residues in the region between amino acids 750 and 870 of the NR2B subunit are sufficient to convey the potentiating effect of PS. The schematic representation of the wild-type NR2B, NR2D and chimeras is on the left. The vertical bars correspond to the four hydrophobic domains. Contribution of NR2B and NR2D to the chimeras is depicted in black

and white respectively. The scales at the top indicate the numbers of the amino acid residue in the wild-type subunits. The percent increase in the NMDA/glycine response in the presence of 100 μM PS is indicated on the right. Holding potential: -70mV. Error bars indicate the standard error of the mean (SEM). Numbers adjacent to the error bars indicate the number of oocytes tested.

Figure 36. NMDA and glycine dose-response curves of wild-type NR1a/NR2B and NR1a/χ4 receptors. (A) The dose-response curve for NMDA was determined in the presence of a saturating concentration of glycine (50 μM). The EC_{50} for wild-type NR1a/NR2B (closed circles with the solid line, n = 4) and NR1a/χ4 (open circles with the dotted line, n = 4) are 22 ± 1 μM and 22 ± 1 μM respectively. The Hill coefficients are 1.8 ± 0.2, and 1.6 ± 0.1. (B) The dose-response curve for glycine was determined in the presence of a saturating concentration of NMDA (300μM). The EC_{50} for wild-type NR1a/NR2B and NR1a/χ4 are 0.3 ± 0.02 μM and 0.1 ± 0.02 μM respectively. The Hill coefficients are 1.5 ± 0.1 and 1.2 ± 0.8.

Figure 37. The potentiating effect of PS, but not the inhibitory effect of 3α5βS, is eliminated in receptors containing NR1a/χ4 subunits. Increase or decrease in the NMDA/glycine response in the presence of PS or 3α5βS is indicated as percent change. PS (100 μM) modulates NR1a/NR2B by 107 ± 19% (open bar, n = 5) and NR1a/χ4 by 3 ± 5% (filled bar, n = 8). 3α5βS (100 μM) alters NR1a/NR2B function by –77 ± 1% (open bar, n = 6) and NR1a/χ4 by -81 ± 1% (filled bar, n = 7). Holding potential: -70mV. Error bars indicate the standard error of the mean (SEM).

Figure 38. NR1a/χ10 containing receptors are inhibited by 3α5βS, and not by PS. PS (100 μM) inhibits the NMDA/glycine response in oocytes expressing wild-type NR1a/NR2D (-40 ± 5%, n = 4, open bar). The NMDA/glycine response of NR1a/χ10 is not significantly changed in the presence of PS (- 4 ± 3%, n = 7, filled bar). 100 μM 3α5βS inhibits both NR1a/NR2D (- 85 ± 4%, n = 4, open bar) and NR1a/χ10 (- 81 ± 1%, n = 7, filled bar). Holding potential: -70mV.

Figure 39. Glycine-independent potentiation by spermine is reduced in NR1a/χ4 receptors. Oocytes expressing wild-type NR1a/NR2B (open bar, n = 4) and NR1a/χ4 (filled bar, n = 5) are assayed at saturating concentrations of NMDA (300 μM) and glycine (50 μM). Increase in the NMDA/glycine response in the presence of spermine (100 μM) is indicated as

percent change. Measurements are made in Ba2+ Ringer's solution at pH 7.5. Holding potential: –20mV. Error bars indicate the standard error of the mean (SEM).

Figure 40. Spermine modulation is dependent on pH and the presence of the α-exon, while PS modulation is not. Oocytes expressing wild-type NR1a/NR2B or NR1b/NR2B were assayed at saturating concentrations of NMDA (300 μM) and glycine (50 μM). Measurements are made in Ba2+ Ringer's solution at pH 7.5 and pH 6.6. Percentage increase in the NMDA/glycine response in the presence or absence of 100 μM spermine or 100 μM PS is indicated. Holding potential is –20mV for spermine experiments and –70mV for PS experiments. Error bars indicate the standard error of the mean (SEM).

Figure 41 is a schematic diagram depicting a model of spermine and PS modulation of the NMDA receptor.

Figure 42. The proton sensitivity of NR1a/χ4 containing receptors is reduced compared to wild-type NR1a/NR2B. Dose dependent responses to proton were recorded under saturating concentrations of NMDA (300 μM) and glycine (50 μM). The IC_{50} for proton at NR1a/NR2B is pH 6.7 ± 0.2 (n = 3) and pH 7.6 ± 0.1 (n = 3) at NR1a/χ4. The Hill coefficients for NR1a/NR2B and NR1a/χ4 are 1.0 ± 0.1, and 1.3 ± 0.3, respectively.

Figure 43. The glycine-independent potentiating effect of spermine at NR1/χ4 receptors is rescued at reduced pH. Oocytes expressing wild-type NR1a/NR2B (open bar), NR1b/NR2B (hatched bar), NR1a/χ4 (black bar) and NR1b/χ4 (gray bar) are assayed at saturating concentrations of NMDA (300 μM) and glycine (50 μM). Measurements of NR1/NR2B and NR1/χ4 activity are made in Ba2+ Ringer's solution at pH 7.5 and pH 6.6 respectively. Responses recorded in the presence of 100 μM spermine, NMDA and glycine are compared to the control responses elicited by NMDA and glycine alone. Percent increase in the NMDA/glycine response is indicated. Holding potential: 20mV. Error bars indicate the standard error of the mean (SEM).

Figure 44. The potentiating effect of PS at NR1/χ4 receptors is not rescued at reduced pH. Oocytes expressing wild-type NR1a/NR2B, NR1b/NR2B (open bars) or NR1a/χ4, NR1b/χ4 (filled black bars) are assayed at saturating concentrations of NMDA (300 μM) and glycine (50 μM). pH values of the recording solution are indicated on the bottom of the chart. Responses recorded in the presence of 100 μM PS, NMDA and glycine are compared to the control

responses elicited by NMDA and glycine alone. Percent increase in the NMDA/glycine response is as indicated. Holding potential: 70mV. Error bars indicate the standard error of the mean (SEM).

Figure 45. Sequence alignment of the region that is important for PS potentiation of the NMDAR. PS potentiates the NMDA/glycine response at receptors containing either NR2A or NR2B subunits, while it inhibits those containing NR2C or NR2D. The residues that are different between the two groups (2A/2B and 2C/2D) are highlighted in yellow. Residues in the NR2B are mutated to the amino acids at the corresponding positions in the NR2D.

Figure 46. The potentiating effect of PS is significantly reduced when Q812 in the NR2B subunit is substituted with lysine (hatched bar). Wild-type NR2B, χ4 (filled bar) and all the individual NR2B mutants are each co-expressed with NR1a in oocytes. Responses are recorded in the presence of 100 μM PS, 300 μM NMDA and 50 μM glycine and are compared to the control responses elicited by NMDA and glycine alone. Percent increase in the NMDA/glycine response is indicated. The horizontal bar under the X-axis indicates that the mutants above it are located in the putative fourth transmembrane domain (TM4). The asterisk designates that the reduction in the PS effect is significant ($p < 0.01$, compared to the wild-type receptor by Student's t-test). N.A. (no activity) indicates that NMDA/glycine could not elicit responses from oocytes expressing NR1a and T835V.

Figure 47. Modulation by PS and spermine is reduced in the NR2B mutant, Q812K. The wild-type NR2B subunit, χ4 (filled bar) and the Q812K NR2B mutant (hatched bar) are each co-expressed with NR1a in oocytes. Percent potentiation by PS and spermine are normalized to the response of the wild-type receptor. 100 μM PS (left panel) potentiates NR1a/χ4 by 12 ± 3% (filled bar, $n = 8$, $p < 0.01$) and NR1a/Q812K by 56 ± 5% (hatched bar, $n = 11$, $p < 0.01$) at –70mV. 100 μM spermine (right panel) potentiates NR1a/χ4 by 18 ± 55% (filled bar, $n = 3$, $p = 0.0001$) and NR1a/Q812K by 35 ± 13% (hatched bar, $n = 6$, $p = 0.003$) at –20mV. The asterisk indicates that the reduction in the PS effect is significant when compared to the wild-type receptor (Student's t-test).

Figure 48 is a schematic diagram of the GluR2 subunit of the AMPA receptor.

Figure 49. The region between amino acid residues 548 and 892 of the NR2D subunit is sufficient to convey the inhibitory effect of PS to NR2B containing receptors. The schematic

representation of the NR2B subunit, the NR2D subunit, and chimeras is on the left. The vertical bars correspond to the four hydrophobic domains. Contribution of NR2B and NR2D to the chimeras is depicted in black and white respectively. The scales at the top indicate the residue numbers in the wild-type subunits. The percent change in the NMDA/glycine response in the presence of 100 μM PS is indicated on the right. Holding potential: -70mV. Error bars indicate the standard error of the mean (SEM). The numbers next to the error bars indicate the number of oocytes tested.

Please insert the following Examples after the Examples at page 78 of the specification:

Example 8. Identification of a Region on the NR2 subunit Conferring Modulation by PS: part II

The domains responsible for the different modulatory effects of PS were mapped in further detail using additional chimeric NR2 subunits. A schematic diagram of the each subunit is shown in Figure 33.

As shown in Example 7 and Figure 31, amino acid residues 870 to 1482 of the NR2B subunit do not mediate the acute modulatory effect of PS at NR1a/NR2B containing receptors. We then asked if the amino acid residues in M4 and the regions adjacent to M4 are important for the modulatory effect of PS. Chimera χ2 was constructed by replacing the region from amino acid residues 750 to 1482 of the NR2B subunit with the equivalent region of the NR2D subunit (774 to 1323).

PS (100 μM) modulation of the NMDA/glycine response in oocytes expressing NR1a/χ2 was eliminated (-6 ± 2%, Fig. 24), suggesting that the necessary residues for the potentiating effect of PS reside within the region between amino acid residues 750 and 870 of the NR2B subunit. Chimera χ3 was constructed to further define the contribution of the region from amino acid residues 750 to 870 of the NR2B . PS (100 μM) potentiation of the NMDA/glycine response was eliminated in oocytes expressing NR1a/χ3 containing receptors (1 ± 3%, Fig. 34). The result confirms our previous conclusion that the residues necessary for the potentiating effect of PS reside within the region between amino acid residues 750 and 870 of the NR2B subunit.

To further localize the necessary components for the potentiating effect of PS, the region from amino acid residues 750 to 870 was subdivided into two parts: amino acid residues 750 to 839 and amino acid residues 840 to 870. Chimera χ4 was constructed by replacing the region between 750 and 839 of NR2B with the corresponding region of NR2D. Similarly, χ5 was constructed by replacing the region between 840 and 870 of NR2B with the corresponding region of NR2D.

The potentiating effect of PS was eliminated in oocytes expressing NR1a/χ4 (-3 ± 5%), whereas the effect of PS in oocytes expressing NR1a/χ5 was not significantly different from that of oocytes expressing wild-type NR1a/NR2B. This finding suggests that the potentiating effect of PS at NR1a/NR2B containing receptors is not dependent on residues 840 to 870 of the NR2B subunit.

We next investigated whether the potentiating effect of PS is dependent on both M4 and the 68 amino-acid region preceding M4 of the NR2B subunit. Chimera χ6 was constructed by replacing the region from residues 750 to 818 of χ3 with the corresponding region of NR2B. Likewise, chimera χ7 was constructed by replacing the region from residues 750 to 818 of NR2B with the corresponding region of the NR2D.

The potentiating effect of PS in oocytes expressing NR1a/χ6 was significantly reduced (38 ± 10%), but not abolished as seen in NR1a/χ3 or NR1a/χ4 (Fig. 34). Thus, replacing M4 alone is not sufficient to eliminate the potentiating effect of PS. However, there are no functional receptors when χ7 is co-expressed with an NR1a subunit. Thus, we are not able to determine whether replacement of the region (750-818 of NR2B) alone would remove PS potentiation.

To rule out the possibility that post-translational modification of the intracellular C-terminal domain of NR2B is involved in the acute potentiating effect of PS, a truncated NR2B subunit (NR2BΔ) was constructed. NR2BΔ was made by introducing a stop codon at amino acid residue 850 in the intracellular domain of NR2B. The only potential phosphorylation site left in the C-terminal domain after the truncation (Y843) was then substituted with alanine to construct NR2BΔ-Y843A.

PS positively modulated the NMDA/glycine response in oocytes expressing NR1a/(NR2BΔ-Y843A) by 83± 16% (Fig. 34). This result supports our previous conclusion that the intracellular domain of the NR2B subunit is not involved in acute PS potentiation.

Furthermore, we conclude that phosphorylation of the NR2B C-terminal intracellular domain is not responsible for the acute potentiating effect of PS. Taken together, the results of these studies indicate that the necessary residues for the acute potentiating effect of PS at NR2B containing receptors reside in both M4 (819 to 839) and the 68 amino-acid region (750 to 818) in the second extracellular domain immediately preceding M4.

Next, we investigated whether the region we have identified above that is necessary for the potentiating effect of PS, NR2B (750-870), is also sufficient to convey the potentiating effect to receptors containing NR2D. We generated an additional chimeric subunit, χ8, by replacing the region from amino acid residues 774 to 892 of NR2D with the corresponding region of NR2B (residues 750 to 870). When co-expressed with the NR1a subunit in oocytes, χ8 containing receptors were positively modulated by PS (72 ± 6%, Fig. 35). Thus, amino acid residues in 750 t0 870 of NR2B are sufficient to confer PS potentiation to receptors containing the NR2D subunit.

We further divided the putative Steroid Modulatory Domain (SMD) into two parts at amino acid residue 842. Chimera χ9 was constructed by replacing residues 842 to 892 of NR2D with the corresponding region of NR2B (818 to 870). χ10 was constructed by substituting residues 774 to 841 of NR2D with residues 750 to 817 of NR2B. PS did not potentiate the NMDA/glycine response at receptors containing either chimeric subunit. PS inhibited the NMDA/glycine response in oocytes expressing NR1a/χ9 by 16 ± 2% and NR1a/χ10 by 4 ± 2%. These results suggest that M4 and the 68 amino-acid region preceding M4 of the NR2B subunit are both required to confer potentiation.

Two chimeric NR2 subunits, χ4 (Fig. 34) and χ10 (Fig. 35), when expressed with the NR1a subunit in oocytes, form receptors that are insensitive to PS. We used these two chimeras to answer the following questions: (1) Do PS and 3α5βS modulate receptors containing NR1a/NR2B subunits by the same mechanism? (2) Does inhibition by PS and 3α5βS reflect a similar mechanism of action in receptors containing NR1a/NR2D? and (3) Do other modulators of the NMDA/glycine response share the same mechanism as PS?

Do PS and 3α5βS modulate receptors containing NR1a/NR2B by the same mechanism?

PS positively modulates NR1/NR2A and NR1/NR2B subtypes of the NMDAR, and 3α5βS negatively modulates all NMDAR subtypes. Using competition assays, PS and

epipregnanolone sulfate (3β5βS) have been shown to modulate the native NMDAR through distinct sites on chick spinal cord neurons and on recombinant receptors expressed in Xenopus oocytes (Park-Chung, Wu et al. 1997). To confirm our previous finding, the effects of PS and 3α5βS were tested at the PS-insensitive chimeric receptor, NR1a/χ4. If PS and 3α5βS modulate the NMDAR via the same mechanism, the inhibitory effect of 3α5βS at NR1a/χ4 should be attenuated.

First, the dose-response curves of agonist (NMDA) and co-agonist (glycine) for NR1a/χ4 receptors were constructed to ensure that the concentrations used in our study are at receptor saturation. The EC_{50} of NMDA or glycine for the receptors containing NR1a/χ4 was not significantly different from that of wild-type receptors (Fig. 36). These results indicate that the agonist and co-agonist binding sites have not been significantly altered and the concentrations of NMDA (300μM) and glycine (50μM) are at saturation.

Next, we compared the effects of PS and 3α5βS at NR1a/χ4 and NR1a/2B. The potentiating effect of PS is abolished in NR1a/χ4 receptors, while the inhibitory effect of 3α5βS is identical to that of wild-type receptors (Fig. 37). Therefore, consistent with the results from competition assays (Park-Chung, Wu et al. 1997), PS and 3α5βS use distinct mechanisms to modulate the NR1a/NR2B subtype of the NMDAR.

Does inhibition by PS and 3αα5ββS reflect a similar mechanism of action in receptors containing NR1a/NR2D subunits?

Both PS and 3α5βS negatively modulate NR1a/NR2D subtypes of the NMDAR in a voltage independent manner. We used the PS-insensitive chimera, χ10, that has an NR2D backbone (Fig. 35), to investigate if loss of PS sensitivity at the receptor would have an influence on the effect of 3α5βS. Saturating concentrations of agonist and coagonist were applied to oocytes expressing NR1a and χ10 subunits. The inhibitory effect of PS was abolished at receptors containing NR1a/χ10, while the inhibitory effect of 3α5βS is retained (Fig. 38). The inhibitory effect of 3α5βS at receptors containing NR1a/χ10 (-85 ± 4%) was indistinguishable from that of receptors containing wild-type NR1a/NR2D (-81 ± 1%). Taken together, these results suggest that PS and 3α5βS exert inhibitory effects at NR1a/NR2D receptors through different mechanisms.

Example 9. Differential Effects of Non-PS Modulators on NMDA Receptors

Next, we investigated whether other modulators of the NMDA/glycine response share the same mechanism as PS. The response of the NMDAR can be modulated by several other endogenous substances, i.e. Zn2+, H+, and polyamines. The PS-insensitive chimera, χ4, was used to investigate whether the domain we identified previously (Fig. 34) is also essential for the modulatory effects of other agents. Polyamines have multiple effects on NMDAR function. At least four different modes of spermine modulation have been identified: (1) glycine-independent potentiation, (2) glycine-dependent potentiation, (3) decrease in affinity for glutamate or NMDA and (4) voltage-dependent blockade of the channel pore (Williams 1997). The receptors containing NR2B subunits and NR1 subunits lacking the N-terminal α-exon are the most sensitive to spermine (Durand, Bennett et al. 1993; Williams, Zappia et al.1994).

To determine whether PS and spermine potentiate NMDAR function by the same mechanism, the glycine-independent form of spermine potentiation at NR1a/χ4 containing receptors was examined. The effect of spermine on the potency of NMDA and glycine was eliminated by performing experiments in the presence of a saturating concentration of agonists (300 μM NMDA and 50 μM glycine). To minimize the voltage-dependent block by spermine, oocytes were voltage-clamped at -20mV.

Spermine (100 μM) potentiated the NMDA/glycine response in oocytes expressing NR1a/NR2B by 143 ± 8% and in those expressing NR1a/χ4 by 20 ± 6% (Fig. 39). The glycine-independent potentiation of spermine at NR1a/χ4 is significantly reduced. The results suggest that spermine and PS may share a common mechanism of action in the modulation of NMDAR function. However, the modulatory effects of spermine and PS at the NMDAR are very distinct.

Native NMDARs in neurons of the forebrain and cerebellum are tonically inhibited by protons, with an IC_{50} (pH 7.3) near physiological pH value (Traynelis and Cull-Candy 1990; Traynelis and Cull-Candy 1991). Protonation of the NMDAR stabilizes a ligand bound closed state. Spermine potentiates the receptor by interacting with the "proton sensor" on the receptor to relieve this tonic proton inhibition (Traynelis, Hartley et al. 1995). The potentiating effect of spermine is enhanced when the pH value of the extracellular space falls; the effect is reduced when the receptor is deprotonated at more basic pH. At pH 7.5, spermine (100 μM) potentiated the NMDA/glycine response of NR1a/NR2B containing receptors by 94 ± 13% (n = 4). When

the pH is lowered to pH 6.6, the potentiating effect of spermine was dramatically increased (323 ± 57%, n = 4, Fig. 40).

The potentiating effect of spermine is also regulated by the presence of the α-exon of the NR1 subunit. The α-exon (exon 5) is located in the N-terminal extracellular domain of the NR1 subunit. It is believed to act as a "tethered" spermine-competitive ligand by binding near the spermine recognition site. The inclusion of the α-exon in the NR1 subunit reduces the spermine sensitivity of the receptor (Durand, Bennett et al. 1993; Traynelis, Hartley et al. 1995). We co-expressed the NR2 subunit with NR1b (the isoform of NR1a that contains the α-exon) to assess the influence of the α-exon on the glycine-independent potentiating effect of spermine. At pH 7.5, the potentiation by spermine at receptors containing NR1b/NR2B was abolished (–6 ± 5%, n = 6, Fig. 40). At pH 6.6, the spermine effect at receptors containing the NR1b splice variant was also significantly reduced (144 ± 25%, n = 6), compared to the effect at receptors containing the NR1a splice variant (323 ± 57%, Fig. 40).

In contrast to the potentiating effect of spermine, the potentiating effect of PS shows little pH and α-exon dependency. At pH 7.5, PS potentiated the NMDA/glycine response of the receptors containing NR1a/NR2B (135 ± 13, n = 8) and the response of those containing NR1b/NR2B (112 ± 11, n = 11) to a similar extent. At pH 6.6, the potentiating effect of PS at receptors containing NR1a/NR2B (137 ± 17, n = 3) and those containing NR1b/NR2B (175 ± 27, n = 3) were also not significantly different ($p > 0.3$). There was no significant difference ($p = 0.62$) between the effect of PS at NR1a/NR2B containing receptors at pH 7.5 and the effect of PS at pH 6.6 (Fig. 40). The effects of PS at NR1b/NR2B containing receptors at these two levels of pH are also not significantly different from each other ($p = 0.10$, Fig. 40).

Thus, the potentiating effects of PS and spermine are very distinct. PS and spermine modulate the NMDAR via two different routes: spermine modulation is coupled to the gating mechanism through the proton sensor, whereas PS modulation occurs via a route that is independent of the receptor's level of protonation (Figure 41). The loss of both PS and spermine modulation of the NMDA/glycine response in receptors containing NR1a/χ4 suggests that these two different routes may converge in a common pathway that modulates the channel gating mechanism. Moreover, the domain we identified at the NR2B subunit (750 – 839) may be an important structural determinant of this common pathway.

We compared the proton sensitivity of wild-type NR1a/NR2B with that of NR1a/χ4. Dose-response curves for proton were constructed (Fig. 42). The IC_{50} (or pKa) for NR1a/NR2B containing receptors is pH 6.7 ± 0.2 and the IC_{50} (or pKa) for NR1a/χ4 is pH 7.6 ± 0.1. NR1a/χ4 receptors are about one magnitude less sensitive to proton than the wild-type receptors. Thus, at the same pH, NR1a/χ4 is less protonated than the wildtype. The reduced level of receptor protonation may be responsible for the fact that NR1a/χ4 receptors were not sensitive to spermine at pH 7.5 (Fig. 39).

We hypothesize that if the spermine-recognition site is still intact, spermine modulation could be rescued by restoring the protonation level of NR1a/χ4 to that of the wild-type receptor. To test this idea, we compared the effect of spermine at chimeric and wild-type receptors at pH values close to their IC_{50} for proton inhibition: pH 6.6 for NR1a/χ4 and pH 7.5 for NR1a/NR2B. At pH 7.5, spermine potentiated responses in NR1a/NR2B by 94 ± 13% (open bar, n = 4). At pH 6.6, spermine potentiated NR1a/χ4 by 94 ± 7%, which is indistinguishable from the response of the wild-type receptor at pH 7.5 (Fig. 43, filled black bar, n = 3). Therefore, by providing equal levels of protonation to the receptors, NR1a/χ4 and NR1a/NR2B containing receptors display a similar degree of sensitivity to spermine. Taken together, our results suggest that the spermine recognition site is most likely not altered in the receptors containing NR1a/χ4.

To provide additional evidence that the spermine recognition site is still functional in NR1/χ4 containing receptors, we investigated whether the presence of the α-exon is still able to regulate the potentiating effect of spermine in NR1a/χ4. NR2 subunits are co-expressed with either NR1b (an NR1 splice variant containing the α-exon) or NR1a (the isoform of NR1b without the α-exon) and the influence of the α-exon on the glycine-independent potentiating effect of spermine is assessed. Both the NR2B and χ4 subunits, when co-expressed with NR1b, render receptors with reduced sensitivity to spermine. At pH levels that are close to the pKa of the NR1b/NR2B (pH 7.5) and NR1b/χ4 receptors (pH 6.6), application of spermine (100 μM) had little or no effect at NMDARs containing NR1b/NR2B (–6 ± 5%, n = 6, Fig. 10, hatched bar) and NR1b/χ4 (12 ± 9%, n = 4, Fig. 43, filled gray bar). Therefore, the α-exon is still capable of attenuating spermine potentiation by competing with spermine at the putative spermine recognition site in receptors containing NR1/χ4.

In conclusion, replacement of M4 and the 68 amino-acid region preceding M4 of the NR2B subunit with the corresponding domain of NR2D decreases the sensitivity of the receptor to protons but does not alter the characteristic properties of the spermine recognition site at the NMDAR.

In contrast to the potentiating effect of spermine on NMDAR function, a decrease in extracellular pH does not rescue the potentiating effect of PS at the receptors containing NR1/χ4 (Fig. 44). At pH 7.5, PS potentiated wild-type NR1a/NR2B responses by 135 ± 13% (n = 8) and NR1b/NR2B responses by 112 ± 11% (n = 11), while PS potentiated NR1a/χ4 by 15 ± 9% (n = 3) and NR1b/χ4 by 24 ± 8% (n = 4, Fig. 44). When measured under more acidic conditions (at pH 6.6), PS potentiation was still not restored at NR1a/χ4 (-13 ± 4%, n = 3) or at NR1b/χ4 (-0.2 ± 6%, n = 3, Fig. 44). Clearly, PS is not able to potentiate responses in receptors containing either NR1a/χ4 or NR1b/χ4 at these two pH levels. Therefore, the loss of PS modulation at receptors containing NR1a/χ4 cannot be a consequence of an alteration in the protonation level of the NMDAR. This result is consistent with our previous findings that the potentiating effect of PS is not dependent on the proton sensor of the NMDAR.

In summary, the region that we have identified (M4 and the 68 amino-acid region preceding M4 of the NR2B subunit) is an important structural determinant for transducing the modulatory signals from the proton sensor of the NMDAR to the gating mechanism. In fact, our report is the first to identify this region of the NR2B subunit as an important domain for proton sensitivity of the NMDAR. In addition, this same region is a necessary requirement for PS modulation, even though the effects of PS are independent of the protonation state of the receptor. Whether the loss of PS modulation reflects an alteration in the PS recognition site, however, still remains to be demonstrated.

Example 10. Structural Analyses of the NMDA Receptor.

In order to identify the individual residues in the region of interest of the NR2B subunit (750 – 839) that play the most important role in the potentiating effect of PS, the amino acid sequences of the four NR2 subunits were compared (Fig. 45). The region under study is highly conserved between the subunits. Because PS potentiates responses in receptors containing the NR2A or the NR2B subunit and inhibits those containing the NR2C or the NR2D subunit, the

residues essential for the differential modulation are most likely to be those that are different between the 2A/2B and 2C/2D subtypes. Fifteen residues were identified and those specific to the NR2B subunit were individually substituted with the equivalent residues of the NR2D subunit (Fig. 45). All of the mutated NR2B subunits were co-expressed with NR1a and modulation by PS was compared to that of the wild-type NR1a/NR2B under saturating concentrations of agonists. Of the 15 mutants tested, none of them showed an elimination of PS modulation as seen in receptors containing χ4 (Fig. 46). However, substituting glutamine at residue 812 with lysine (Q812K) significantly reduced the potentiating effect of PS ($p < 0.01$, Fig. 46 and Fig. 47, hatched bar). The EC_{50} for NMDA or glycine at receptors containing NR1a/NR2B (Q812K) is not different from wild-type receptors. For receptors containing NR1a/NR2B (Q812K), the EC_{50} of NMDA is 20.7 ± 4.2 μM (n = 4) and the EC_{50} of glycine is 0.2 ± 0.007 μM (n = 4). For wild-type receptors, the EC50 of NMDA is 20.1 ± 3.5 μM (n = 4) and glycine EC_{50} is 0.2 ± 0.005 μM (n = 4). Mutant receptors containing Q812K also displayed a significant reduction in the glycine-independent potentiating effect of spermine (Fig.47, right panel). Thus, residue Q812 is important for both the potentiating effect of PS and spermine. Nevertheless, PS modulation is not abolished in the mutated receptor (Q812K), suggesting that there are additional residues that play a key role, and it may be necessary to mutate them along with Q812K in order to completely eliminate PS modulation. This result is not unexpected given the fact that replacement of both the 68 amino-acid region preceding M4 and M4 concurrently is required to eliminate the effect of PS (see results for χ3 and χ6 in Fig. 34).

To propose a structural model of our region of interest in the NMDAR, we compared this region to the crystal structure of the glutamate binding domain of the GluR2 subunit of the AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor (Armstrong, Sun et al. 1998). The crystal structure consists of the second extracellular domain and ~150 residues amino-terminal (S1) to the first transmembrane domain on the GluR2 subunit (Fig. 48). This region is conserved among the family of glutamate receptors. All the glutamate receptors share this core structure. The amino acid sequence of our region of interest that is essential for the potentiating effect of PS is compared with that of GluR2 below.

The first 54 amino acids are included in the GluR2 crystal structure (underlined above). The remaining portion of the sequence containing M4 and the linker region between the

glutamate binding domain and M4 remains to be described. The first 54 amino-acid region of the GluR2 subunit contains two solvent-exposed helices, J and K (Fig. 48). Several residues in this region have been shown to be involved in regulation of ligand binding and gating kinetics in the AMPAR (Armstrong, Sun et al. 1998). The RNA editing site and all of the alternative splice sites (flip/flop) are located in this region. The flip and flop variants of the AMPAR differ in their desensitization kinetics and sensitization to different modulators. Cyclothiazide, aniracetam and thiocyanate are proposed to modulate AMPAR activity through this domain (Partin, Bowie et al. 1995; Partin, Fleck et al. 1996). In addition, the autoantibodies found in patients with Rasmussen's encephalitis that cause constitutive activity of the AMPAR binds to the epitope located just upstream from the J helix (Rogers, Andrews et al. 1994). Thus, this region is important for regulating the channel activity of the AMPAR. We used a computer program, SwissPdbViewer 3.6 to thread the NMDAR sequences through the crystal structure of the GluR2 subunit. We can conduct more detailed modeling experiments in the future in order to build a model that can correlate differences in the structures of NR2 subunits with differences in their responses to PS and protons. Our studies have shown that the J/K helices, along with the linker region and M4 are important structural determinants for the modulatory effects of protons and of PS on NMDAR channel activity. It is certainly interesting that this same region in GluR2 has been shown to play a role in the allosteric modulation of receptor function.

The inhibitory effect of PS at receptors containing NR1a/NR2D subunits.

We have identified a region of the NR2B subunit (750 – 870) that is necessary for PS potentiation (Fig. 34). We also have reported that this same domain is sufficient to convey PS potentiation to the receptors containing the NR2D subunit (Fig. 35). The aim of the next set of experiments was to produce chimeric receptors that will help define the minimal domain of the NR2D subunit that is responsible for inhibition by PS. The NR2D subunit was used as the backbone to construct two NR2D-NR2B chimeras: χ11 and χ12 (Fig. 49). First, the N-terminal region of the NR2D subunit (amino acid residues 1 to 548) was replaced with the corresponding region on the NR2B subunit (amino acid residues 1 to 524) to construct χ11. Next, the C-terminal region of χ11 (amino acid residues 892 to 1323) was replaced with the corresponding region on the NR2B subunit (amino acid residues 870 to 1482) to construct χ12.

PS inhibited the NMDA/glycine response in oocytes expressing either NR1a/χ11 (36± 7%) or NR1a/χ12 (47 ± 9%, Fig. 49). In fact, there was no significant difference between wild-type NR2D containing receptors (43 ± 11%) and the chimeras in terms of their inhibitory response to PS. Therefore, the region from amino acid residues 548 to 892 of NR2D is sufficient to convey PS inhibition.

To further localize the domain important for the inhibitory effect of PS, the region (548 to 892) of NR2D was divided into two parts at residue 774 (Fig. 49). Chimera χ13 was constructed by replacing residues 524 to 750 of NR2B with the corresponding region of NR2D. Chimera χ14 was constructed by substituting residues 750 to 870 of NR2B with residues 774 to 898 of NR2D. PS (100μM) did not inhibit the NMDA/glycine response at receptors containing either chimera (Fig. 49). Thus, residues surrounding 774 of NR2D are required to establish PS inhibition at chimeric receptors that are constructed using NR2B as the backbone. Chimera χ15 (Fig. 49) can be used to determine whether the first three hydrophobic membrane domains are required for this inhibitory effect of PS at NR2D containing receptors.

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